Animal Biotechnology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597228

Enhanced Host Immune Recognition of Mastitis Causing *Escherchia Coli* in CD-14 Transgenic Mice

R. Wall [a]; A. Powell [a]; E. Sohn [b]; J. Foster-Frey [a]; D. Bannerman [c]; M. Paape [c]
[a] Animal Bioscience and Biotechnology Laboratory, Agricultural Research Service, Beltsville, Maryland, USA [b] Genetic Disease Resistance Section, NIDDK, National Institutes of Health, Bethesda, Maryland, USA [c] Bovine Functional Genomics Laboratory, Agricultural Research Service, Beltsville, Maryland, USA

To cite this Article Wall, R., Powell, A., Sohn, E., Foster-Frey, J., Bannerman, D. and Paape, M.(2009) 'Enhanced Host Immune Recognition of Mastitis Causing *Escherchia Coli* in CD-14 Transgenic Mice', Animal Biotechnology, 20: 1, 1 — 14
To link to this Article: DOI: 10.1080/10495390802594206
URL: http://dx.doi.org/10.1080/10495390802594206

Animal Biotechnology, 20: 1–14, 2009
ISSN: 1049-5398 print/1532-2378 online
DOI: 10.1080/10495390802594206

Taylor & Francis
Taylor & Francis Group

ENHANCED HOST IMMUNE RECOGNITION OF MASTITIS CAUSING *ESCHERCHIA COLI* IN CD-14 TRANSGENIC MICE

R. Wall[1], A. Powell[1], E. Sohn[2], J. Foster-Frey[1], D. Bannerman[3], and M. Paape[3]

[1]*Animal Bioscience and Biotechnology Laboratory, Agricultural Research Service, Beltsville, Maryland, USA*

[2]*Genetic Disease Resistance Section, NIDDK, National Institutes of Health, Bethesda, Maryland, USA*

[3]*Bovine Functional Genomics Laboratory, Agricultural Research Service, Beltsville, Maryland, USA*

Escherchia coli ***causes mastitis, an economically significant disease in dairy animals.*** **E. coli** ***endotoxin (lipopolysaccharide, LPS) when bound by host membrane proteins such as CD-14, causes release of proinflammatory cytokines recruiting neutrophils as an early, innate immune response. Excessive proinflammatory cytokines causes tissue damage, compromising mammary function. If present, soluble CD-14 (sCD-14) might out compete membrane bound CD-14, lessening the severity of the inflammatory response. To test this hypothesis transgenic mice, expressing sCD-14 in their milk (31 to 316 μg/ml), were evaluated. A cell culture study demonstrated, in the presence of LPS, milk from transgenic mice increased secretion of cytokine IL-8 compared to milk from nontransgenic littermates (18 ± 3 vs. 35 ± 2 ng/mL, $p < 0.001$). To assess protection afforded by the transgene, glands were infused with*** **E. coli.** ***Recovery of bacteria showed no clear relationship between sCD14 concentration and the number of organisms recovered; however, there was a strong relationship between sCD14 concentration and edema ($r^2 = 0.999$, $p < 0.001$), as measured by weight of fluid in harvested glands. Highest expressing lines had the least edema, suggesting the presence of sCD14 had an effect on reducing the inflammatory response to*** **E. coli,** ***thus, possibly protecting against gland tissue damage.***

Keywords: CD-14; Mammary gland; Mastitis; Transgenic mice

Mastitis, which is a very costly and prevalent disease in the dairy industry, decreases the quality and quantity of milk produced, and has a negative impact on animal well-being and longevity. Mastitis is most commonly caused by a bacterial infection of the mammary glands. *Escherchia coli* is among the most common bacteria causing clinical cases of mastitis (1). Although implementation of

The manuscript is dedicated to the memory of Leah Schulman, an invaluable member of the team. Raymond Fetterer is thanked for his assistance with Western blot analysis.

Address correspondence to R. Wall, Bldg. 230, Rm 101, Barc-East, Beltsville, MD 20705, USA. E-mail: Bob.Wall@ARS.USDA.GOV

udder and milking parlor hygiene have greatly reduced the incidence of mastitis caused by contagious pathogens over the past half century, these practices have had a negligible effect on the cases of mastitis caused by environmental pathogens, such as *E. coli*.

The surface of Gram-negative bacteria, such as *E. coli*, is decorated with LPS, also referred to as endotoxin. The innate immune system is readily poised to detect LPS and respond to Gram-negative bacterial infections. LPS binding protein (LBP) and CD-14, among other accessory binding molecules, facilitate LPS activation of Toll-like receptor 4 (TLR4) and corresponding up-regulation of expression of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukins (IL-1, IL-6, and IL8). These cytokines, in turn, contribute to the recruitment and activation of neutrophils, which are among the first leukocytes to defend the host against the offending bacteria. Excessive local release of proinflammatory cytokines, including TNF-α, in response to LPS can cause local tissue damage, whereas excessive systemic release can lead to toxic shock, which, under severe instances, can be fatal.

CD-14 is a phosphatidylinositol-anchored protein found on monocyte, macrophage, and neutrophil membranes. It also exists in a soluble form (sCD14), which is thought to be derived from macrophage and neutrophil membranes and/or directly exocytosed from these cells. It appears that the soluble and membrane bound forms of CD-14 compete for binding of LPS. As a result, elevated concentrations of sCD14 can modulate the humoral and cellular responses otherwise triggered when LPS and associated proteins bind membrane bound CD14 and interact with TLR4. Furthermore, it has been shown that sCD14 and LBP can bind LPS and transport it to high-density serum lipoproteins thus reducing (detoxifying) LPS serum levels (2). The protective nature of sCD14 has been demonstrated in challenge studies in mice (3, 4) and cows (5, 6) in which exogenous sCD14 was supplied. Also, transgenic mice expressing elevated levels of CD-14 on monocytes, neutrophils, and lymphocytes have been shown to be highly sensitive to LPS (7), whereas transgenic mice overexpressing sCD14 are protected from fatal toxic shock (8).

Based on the demonstrated ability of sCD14 to attenuate innate immune response to LPS exposure, we hypothesized that increasing the concentration of sCD14 in mammary glands would increase immune surveillance sensitivity and, thus, modulate the immune response that is operative during mastitis. sCD14 concentration can be increased by introducing a transgene encoding sCD14 expressed in mammary gland epithelium during lactation. We have previously demonstrated that expressing an antimicrobial peptide, lysostaphin, in mammary gland epithelium can protect mice and cows from mammary gland infections caused by *Staphylococcus aureus* (9, 10). Though highly effective, persistent expression of such an antimicrobial peptide may eventually lead to evolution of resistance by *S. aureus* and other targeted organisms. In contrast, increasing the ability of innate immunity to combat a microorganism by enhancing host immune recognition of a core conserved molecule on the pathogen (e.g., LPS) would be less likely to result in drug resistance; therefore, to test our hypothesis we generated, characterized, and challenged four transgenic mouse lines overexpressing sCD14 in their mammary glands during lactation.

METHODS

Generation of CD–14 Transgenic mice

The 4.2 Kb 5′-flanking region with the first 29 bases of the untranslated region and 2.1 Kb of the 3′-flanking region of the ovine beta-lactoglobulin (βLG) gene were obtained from Dr. A. J. Clark (pBJ41; Roslin Institute, UK) and served as the regulatory element for the transgene. The βLG promoter has been successfully used in mice, sheep, and cattle to direct the expression of transgenes to mammary gland secretory epithelium (10–12). The coding sequence of the transgene included a 1 Kb, *EcoRI – NcoI* fragment containing cDNA encoding the N-terminal 358 amino acids of bovine CD14 derived from Holstein lung tissue (13), the hGH signal peptide region, and the bGH polyadenylation signal. Three- to four-week-old C57BL6/SJL mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and housed in the animal facility at the Beltsville Agricultural Research Center. All animal experiments were conducted in accordance with protocols approved by the BARC Institutional Animal Care and Use Committee. At 5 weeks of age females were superovulated and mated, and pronuclear embryos were harvested, microinjected and transferred to pseudopregnant recipients by standard techniques. Offspring were screened for fusion gene incorporation by Southern blot and/or PCR using DNA from tail tips. Southern blots were probed with a ^{32}P-labelled, full-length transgene by standard techniques. For PCR, the primer pair (CD14up1: ctggacggaaatcccttct; CD14down2: ggttctttccgcctcagaag) was designed to generate a 396-bp fragment spanning the junction between the BLG promoter and CD-14 cDNA.

CD-14 Detection in Transgenic Mouse Milk

Females were milked between days 8 and 12 of lactation (14). Litters were removed from dams for 4 hours, females were anesthetized with Avertin (375 μg/g body weight) and oxytocin (150 μL of 20 USP posterior pituitary units/mL, Vedco NDC 50989-420-12) was administered by intraperitoneal injection. Milk was collected by aspiration, weighed, and immediately diluted with PBS, centrifuged for 10 min at 16,000 × *g* and infranatant (whey) was frozen at −20°C. Milk samples to be analyzed by Western analysis, essentially as previously described (15), were diluted 1:2 with 50 mM EDTA, centrifuged for 10 min at 16,000 × *g* and the infranatant frozen at −20°C until the time of analysis. The sCD14 standard used in the Western analysis was diluted in whey from nontransgenic mice. Transgenic whey and control whey samples were heated at 70°C for 10 min with loading buffer. Nupage 4–12% Bis-Tris gels (Invitrogen # NPO 322) were used with MOPS buffer and no reducing agent. Immunoblot-P membrane was used for transfer. Following transfer and before blocking in Pierce 37517 Superblock for 1 hour at room temperature, the portion of membranes containing peptides 60 kDa and smaller were removed to avoid nonspecific probe-binding. Monoclonal antibody (clone CAM36A, VRMD Inc., Pullman, WA), which does not cross react with mouse CD-14, was diluted 1:1,000 and used as first antibody and goat antimouse second antibody (Pierce Cat. # 1858413) was used at 1:500. Digital images of the luminol stained (Pierce Cat. # 34076 supersignal west dura extend duration substrate)

membranes were digitized with ImageQuant software (version 5.2, Molecular Dynamics, Pittsburg, PA). CD-14 standards for the Western blot analysis and ELISAs were produced in a baculovirus system. Recombinant baculovirus containing an expression vector encoding bovine CD14 was generated as previously reported (13). Expression and purification of recombinant bovine CD14 protein using an insect cell system analogous to that previously described above was performed by a commercial vendor (Kemp Biotechnologies, Inc., Frederick, MD, USA).

Mammary Gland Function Analysis

To evaluate the functional integrity of the mammary gland of transgenic dams, milk yield was estimated by the weigh suckle-weigh method (16, 17). Transgenic and nontransgenic dams who did not retain six pups to day 10 of lactation were excluded from the analysis. Milk production was measured between days 9–12 of lactation. On test days litters were separated from their dams for 4 hours. After the separation period litters were weighed, returned to dams to nurse for 2 hours, and then reweighed. Milk yield was determined indirectly from the differences in the litter weights before and after suckling.

Bovine Aortic Endothelial Cell Assay

To assess the biological activity of the sCD14 produced in the milk of transgenic mice an in vitro assay was conducted as previously described (15). The assay is based on IL-8 production by and lysis of endothelial cells in response to binding of LPS by sCD14. Confluent cells in a 96-well plate were washed twice with 100 µl warm PBS. Mouse milk diluted 1:2 in PBS was further diluted in 100 µL of Kaign's media to 1:100 and added to the cells. Wells were either treated with 10 µL of PBS or 10 µL of LPS (10 ng/well, *E. coli* 0111:B4, Sigma L3024). Plates were incubated at 37°C in 5% CO2 for 24 hours, then centrifuged at $250 \times g$ for 10 min. Fifty µL were harvested for the IL-8 assay and the remaining fluid was aspirated from the wells and wells were washed twice with 100 ul of warm PBS. In preparation for measuring protein content, cells were lysed with 50 µL of M-PER mammalian protein extraction reagent (Pierce Cat # 78503), shaken for 5 min at room temp and then incubated at 37°C for 10 min. Twenty-five µL of bicinchoninic acid (BCA) reagent (Pierce BCA assay Cat # 23225) were added to wells and concentrations of the resulting cuprous cation were quantified on a Spectra Max 340 plate reader (Molecular Devices) at 562 nm.

Intramammary Infusions

A preliminary study was conducted to confirm Trypan Blue solution (0.4% Trypan Blue in phosphate buffered saline, Sigma; T-8154) could be used as a marker dye to assess the success of infusions without eliciting an innate immune response. In the preliminary experiment four treatments were compared in wildtype mice: (1) Trypan Blue infusion, (2) LPS (20 µg/mL, Sigma; L3024) infusion, (3) saline infusion, and (4) no infusion. In the subsequent challenge studies glands of

transgenic and nontransgenic littermates from each of the four transgenic lines were infused with *E. coli* (100 CFU/mL), or received no infusion.

On the day of challenge (days 9–12 of lactation) pups were removed from dams for 4 hours. Pups were weighed then returned to their dams and allowed to nurse for at least 1 hour. Pups were again weighed when removed from dams at the time of challenge. After lightly anesthetizing females with Avertin, abdominal glands were infused. In the preliminary study left side glands L3 and L4 were infused with saline and Trypan Blue solution respectively and right side glands R3 and R4 received no treatment and LPS infusion respectively. When the *E. coli* challenge was performed glands L3 and L4 received *E. coli* infusions, the distal end of R3 was snipped but otherwise the gland was not treated and gland R4 remained untreated.

Infusions were performed following removal of approximately 0.5 mm of the distal end of teats and threading a 32 g luer-lock stub adapter (LSA-32, Access Technologies, division of Norfolk Medical, Inc., Skokie, IL, USA) attached to a 50-μL Hamilton syringe, into the teat canal approximately 4 mm. The syringe contained approximately 5 CFU of *E. coli* in 50 μL of Trypan Blue solution. Preliminary experiments had demonstrated that survival rates of *E. coli* incubated in Trypan Blue solution or PBS were not different (data not shown). Following infusions, surgical glue (Nexaband SC 603–3730) was placed on all snipped glands to prevent leaking, infection, or crosscontamination. After 18 hours mice were anesthetized with Avertin, injected IP with oxytocin and pectoral glands R1 and L1 gland were milked. Animals were then euthanized by cervical dislocation and mammary glands were dissected and weighed. A portion of the gland was homogenized (IKA Ultra-Turrax T25, Cole-Parmer) in a volume of PBS to achieve 100 mg of gland per milliliter of solution. Care was taken to decontaminate the homogenizer blades between glands by washing in diluted Roccal and repeated rinses with 70% ethanol. The final water washes were plated to confirm minimal contamination.

An aliquot of the resulting homogenates were serial diluted and duplicate plated on TSA. Plates were incubated at 37°C for at least 18 hours and counted by hand or with the aid of a plate reader (AES Chemunex Laboratorie, Paris, France) as was appropriate for the density of the colonies. An aliquot of the homogenate was also taken for DNA concentration estimations and the remaining homogenate was centrifuged at $16{,}000 \times g$ for 30 min and the infranatant frozen at −20°C until thawed for use in cytokine assays including TNF-α, KC, and MIP-2.

DNA Concentration

DNA concentration was determined from homogenized mammary gland samples with the aid of a nanodrop analyzer (Nanodrop ND1000, Thermo Fisher Scientific, Waltham, MA).

Cytokine Assays

Enzyme-linked immunosorbent assays (ELISA) were performed per manufacturer's recommendations. Samples for TNF-α determinations were analyzed undiluted (R&D Systems, Cat. # MTA00). Samples from challenged mammary glands for KC (R&D Systems, Cat. # MKC00B) and MIP-2 (R&D Systems, Cat. #

MM200) assays were diluted 1:400 while the samples from control glands were diluted 1:10.

Wet-dry weights. To estimate the tissue fluid content, infused mammary glands were weighed immediately after dissection (Ohaus oven GA200D), then oven dried at 100°C for 18 hours and then reweighed to record dry weight.

Stats. General linear model (GLM) analysis was conducted using SPSS version 13.0 for Windows software (SPSS Inc., Chicago, IL, USA). GLM models always included Line, Genotype, and Treatment as main effects and all two-way interactions. Days in milk, litter size, and suckling time were included as covariants when appropriate and then excluded from the model, if they did not significantly contribute to explaining variation. Regression analysis was performed using SigmaPlot for Windows version 9.0 software (Systat Software, Inc., Chicago, IL, USA). Least square means and standard error of the means are reported throughout.

RESULTS

Characterization of Transgenic Lines

Four sCD14 expressing transgenic mouse lines were evaluated. Western blot analysis of milk samples (minimum of seven observations per line) from the four transgenic lines revealed sCD14 concentrations ranging from 31 to 316 μg/mL (line 04: 316 ± 37 μg/mL; line 15: 198 ± 44 μg/mL; line 16: 39 ± 48 μg/mL; line 25: 31 ± 50 μg/mL). To assess the influence of transgene expression on functionality of mammary glands, a weigh-suckle-weigh experiment was conducted. Litters were weighed before and after nursing. The difference in weight was taken to be the weight of milk that the pups consumed. The weight of the milk suckled did not differ between lines or from wildtype mice (line 04, 715 ± 124 mg; line 15, 536 ± 121 mg; line 16, 576 ± 136 mg; line 25, 691 ± 128 mg; wildtype, 782 ± 205 mgi $p = 0.246$). Also, milk weights were not influenced by genotype (transgenics, 633 ± 60 mg; non-transgenic littermates, 668 ± 75 mgi $p = 0.683$).

To verify the sCD14 produced by the transgenic mice was biologically active, transgenic mouse milk from the four transgenic lines was added to bovine aorta endothelial cell cultures and IL-8 production and protein loss were measured in the presence and absence of LPS. This assay is based on the premise that LPS bound by CD14 elicits an innate immune response (IL-8 production) and cell detachment and lyses endothelial cells (measured by protein loss). The results from that study are presented in Figure 1 and Table 1.

The addition of serum, a source of sCD14, or LPS alone to endothelial cell cultures had no discernable affect on the apparent health of the cultures and appearance of those treatment groups did not differ from cell culture wells with no additive (Figure 1 panels A vs. B and D). Similarly, transgenic mouse milk, in the absence of LPS, had no apparent detrimental influence on the cell cultures (data not shown). As expected, addition of serum and LPS together resulted in lysis of endothelial cells (Figure 1, panel E). Milk from a transgenic mouse plus LPS had a similar detrimental effect on cell viability as serum plus LPS (Figure 1, panel F).

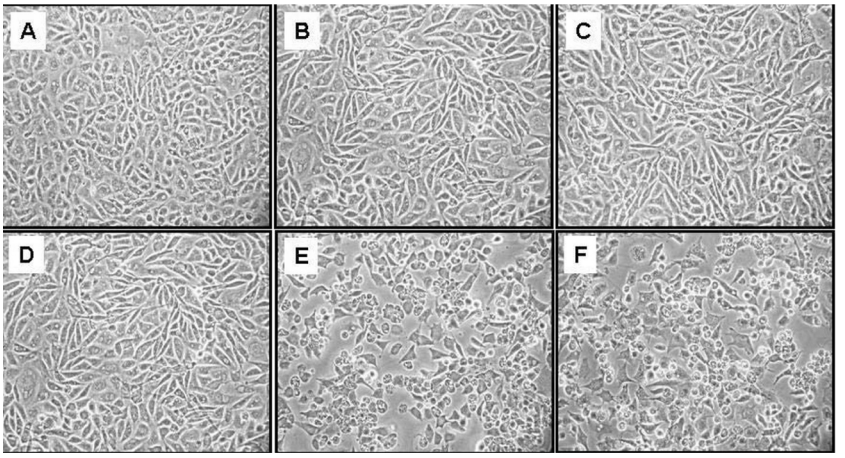

Figure 1 Influence of milk from sCD14 transgenic mice on bovine aorta endothelial cell cultures. (A) No additives; (B) serum only, a source of sCD14; (C) milk from a sCD14 transgenic mouse only; (D) LPS only; (E) serum plus LPS; (F) milk from same mouse as in panel C plus LPS.

There was no difference in IL-8 production by endothelial cells in culture attributable to milk from different transgenic lines (Table 1, $p = 0.854$). But IL-8 production was clearly increased when milk from transgenic animals was added to cultures in the presence of LPS when compared to milk plus LPS from their nontransgenic littermates (17.8 ± 2.6 ng/mL vs. 34.7 ± 1.5 ng/mL, Table 1, $p < 0.001$). Loss of protein in cultures was greatest when milk from transgenic mice was added to cultures in the presence of LPS in comparison to milk from non transgenic littermates ($30 \pm 2\%$ vs. $45 \pm 1\%$, Table 1, $p < 0.001$). But the magnitude of protein loss in the cell cultures did not differ between transgenic lines (Range: $32 \pm 2\%$–$40 \pm 3\%$, Table 1, $p = 0.648$).

Preliminary infusion study. A preliminary experiment was conduced to determine if Trypan Blue could be used as a marker dye to confirm successful mammary gland infusions without eliciting a detectable immune response. The experiment was also designed to determine if harvesting mammary glands 6 or 18

Table 1 IL-8 concentration and protein loss in endothelial cell cultures exposed to milk from four lines of sCD14 transgenic mice and their nontransgenic littermates (mean ± SEM [number of milk samples assayed])[1]

	IL-8 (ng/mL)		Protein loss (%)	
Line	Nontransgenic	Transgenic	Nontransgenic	Transgenic
04	16.3 ± 4.6 (4)	34.3 ± 2.6 (12)	32 ± 5.0 (4)	47 ± 3 (12)
15	15.4 ± 5.3 (3)	34.3 ± 2.6 (12)	33 ± 6 (3)	44 ± 3 (12)
16	11.5 ± 3.7 (6)	35.1 ± 2.4 (14)	20 ± 4 (6)	43 ± 3 (14)
25	20.6 ± 4.1 (5)	35.4 ± 2.2 (17)	33 ± 4 (5)	47 ± 2 (17)

[1]IL-8 was undetectable in cultures without a source of sCD14 both in the presence and absence of LPS. Likewise, no protein loss was detected in wells that did not contain both a source of sCD14 and LPS.

hours post infusion influenced the magnitude of the response measured. Wildtype mice were used in this study and TNF-α concentration and DNA content, normalized for gland weights served as endpoints. TNF-α concentration in mammary glands harvested following infusion of 0.4% Trypan Blue in PBS (17.2 ± 8.3 pg/mL) did not differ from those of saline infused glands (15.7 ± 8.3 pg/mL) or glands that were not infused (13.0 ± 8.3 pg/mL), but all three treatment effects differed from glands infused with 20 ng/μL LPS (68.3 ± 8.3 pg/mL, $p < 0.001$). There was a tendency for TNF-α concentration to be numerically larger at 18 hours post infusion, but the difference was not statistically significant.

DNA content per gram of gland diminished as a result of LPS infusion compared to infusion with saline (1.1 ± 0.1 ng/gm vs. 0.8 ± 0.1 ng/gm, $p = 0.049$). When the DNA content of glands infused with LPS was measured at 6 or 18 hours post infusion, less DNA was detected at 18 hours ($p = 0.016$), suggesting the immune response continues to ravage mammary glands at 18 hours post infusion.

***E. coli* challenge study.** To determine if expression of sCD14 in mammary glands protects them from *E. coli* infections, glands L3 and L4 were infused with *E. coli*, while glands R3 (tip excised but gland not infused) and R4 (no treatment) served as controls. Sixty-three mice were challenge from the four lines. At the time of gland dissection an Outcome score, based on the extent of dye diffusion into glands (0–5, with 5 = complete penetration), was assigned to each infused gland. Nine mice were removed from the analysis because infusion Outcome scores were less than 5. Sixty-three *E. coli* infused transgenic glands and 32 infused glands from nontransgenic littermates were included in the statistical analysis. Sixty-four noninfused or saline infused glands from the same transgenic mice and 38 glands from nontransgenic littermates served as controls. Eighteen hours after infusion, glands were harvested, homogenized, and supernatants plated to enumerate *E. coli* content. Noninfused and saline infused control glands had bacteria counts in the range of 10 to a few hundred organisms (mean = 2.6 ± 0.2 log CFU/mL) and differed significantly from the concentration of bacteria recovered from the infused glands which averaged in the hundreds of millions of organisms (8.0 ± 0.2 log CFU/mL, $p < 0.001$). Transgenic and control mice of different lines responded differently ($p < 0.001$ for line by genotype interaction, Table 2). Recovery of bacteria from transgenic and nontransgenic glands

Table 2 Log CFU per milliliter of *E. coli* recovered from infused mammary glands harvested 18 hours postinfusion[1]

Line	Transgenics	Non-transgenic	p
04	5.7 ± 0.3	3.2 ± 0.4	<0.001
15	5.4 ± 0.3	6.3 ± 0.3	0.056
16	5.3 ± 0.3	5.3 ± 0.4	0.939
25	6.3 ± 0.3	4.9 ± 0.4	0.004

[1]Noninfused control glands from the same mice averaged 2.5 ± 0.2 log CFU/mL.

did not differ for lines 15 ($p = 0.056$) and 16 ($p = 0.939$). Whereas recovered bacteria were greater in transgenic mice for lines 04 ($p < 0.001$) and 25 ($p = 0.004$). TNF-α concentration, normalized for mammary tissue weight, in glands harvested 18 hours after challenge was higher in glands infused with *E. coli* (79 ± 4 ng/gm tissue) compared to those that were not infused with *E. coli* (3 ± 3 ng/gm tissue, $p < 0.001$); however, TNF-α concentration was not influenced by the presence (45 ± 3 ng/gm tissue) or absence (37 ± 4 ng/gm tissue) of the transgene ($p < 0.141$). Furthermore, there was no evidence that mice from different lines responded differently (range: 34 ± 6–49 ± 5 ng/gm tissue, $p = 0.162$).

The two chemoattractants KC and macrophage-inflammatory protein 2 (MIP-2) were measured in harvested glands 18 hours post infection. MIP-2, a chemokine expressed on infiltrating inflammatory leukocytes such as neutrophils, serves as an indirect measure of the neutrophil population in mammary glands following challenge. The presence of *E. coli* resulted in elevated MIP-2 (162.5 ± 10.2 ng/mL) compared to control glands (0.7 ± 0.9 ng/mL, $p < 0.001$); however, as with TNF-α, the MIP-2 response was similar in transgenic and non-transgenic animals (87.1 ± 8.1 ng/mL vs. 76.0 ± 10.7 ng/mL, $p = 0.413$). Lines did not differ in MIP-2 content of their glands (range: 60.1 ± 13.9–91.4 ± 13.9 ng/mL, $p = 0.217$). KC, primarily expressed in fibroblasts and endothelial cells, responded as did TNF-α, and MIP-2. There was a detectable treatment difference (199 ± 12 ng/mL E.coli infused vs. 6 ± 10 ng/mL controls, $p < 0.001$), but genotype did not influence KC levels (77.0 ± 13.2 vs. 75.7 ± 10.2 ng/mL, $p = 0.968$) nor did transgenic mouse lines (range 64.5 ± 14.7 ng/mL–86.1 ± 16.4 ng/mL, $p = 0.606$).

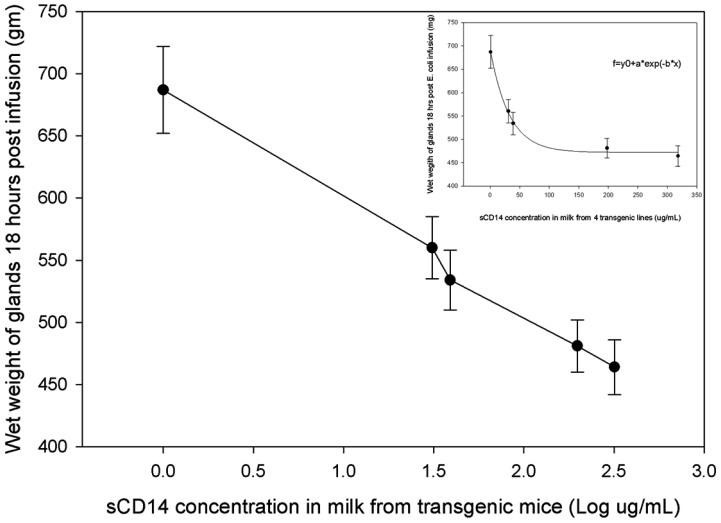

Figure 2 Relationship between wet weight of glands harvested from *E. coli* infused mice and level of sCD14 in the milk of the transgenic mouse lines. sCD14 concentration in milk from transgenic mice (Log μg/mL). Zero sCD14 concentration data point is the mean of weights of all E. coli infused glands of nontransgenic littermates from the four lines infused wildtype mice. Insert shows the natural exponential decay relationship.

Table 3 Wet-dry gland weight difference (gm) in transgenic sCD14 mice infused with *E. coli* at day 10 of lactation

	Genotype		
Treatment	Non-tg (n = 16)	Transgenic (n = 10)	Trt effect (p = 0.002)
Not infused (n = 13)	0.52 ± 0.02	0.45 ± 0.03	0.48 ± 0.02
Infused (n = 13)	0.64 ± 0.02	0.54 ± 0.03	0.59 ± 0.02
Genotype effect (p = 0.014)	0.58 ± 0.02	0.49 ± 0.02	

To measure edema caused by the *E. coli* induced inflammatory response, glands were weighed at the time of harvest, dried overnight, and reweighed. In preliminary experiments it was demonstrated that wet weights of noninfused (0.32 ± 0.03 gm) and saline infused (0.34 ± 0.03 gm) glands did not differ ($p = 0.693$); therefore, control glands were not infused in this study. There was a strong ($r^2 = 0.999$) linear inverse correlation between the wet weight (sum of glands L3 + L4) of *E. coli* infused glands harvested 18 hours postinfusion and the log of sCD14 concentration of milk from the transgenic lines (Figure 2, $p < 0.001$), suggesting less fluid build-up in glands from mice expressing higher levels of sCD14. This observation is further supported by the treatment difference in wet minus dry weights for glands from infused and control treatments (Table 3, $p = 0.002$). Furthermore, it was observed that there was less edema in transgenic mice compared to their littermate controls (Table 3, $p = 0.014$).

DISCUSSION

Based on the hypothesis proposed by Paape and colleagues (6) increasing immune surveillance of the mammary gland could, by quickly eliminating invading organisms, reduce the severity of mastitis. We have tested that hypothesis by assessing the response of mice expressing various levels of sCD14 in their mammary glands to *E. coli* challenge.

Milk is a known source of sCD14 and it is found in bovine (18) and human breast milk (19) at low μg/mL levels. sCD14 is also present in mouse milk (20, 21) though quantitative data has not be reported. The concentration of the transgene product in the four transgenic lines of mice studied here ranged from 31 to 318 μg/mL. These levels compare favorably with concentrations of transgene products in other mouse bioreactor projects which utilized the βLG promoter to drive reporter genes such as alpha-antitrypsin at 46–9000 μg/mL (22), factor XI at 0.3 to 60 μg/mL (23), and lysostaphin at 0.3 to 12 μg/mg protein (9). In spite of the fact that there was an order of magnitude difference in transgene expression levels among the four lines, there did not appear to be a difference in mammary gland function based on milk yield from the weigh-suckle-weigh experiment. That is not always the case for mammary gland specific transgenes. It is not uncommon to observe compromised behavior of mammary gland function attributable to transgene expression (17, 24). The fact that milk yield was similar among lines suggests that high levels of sCD14 in and around mammary epithelium is in and of itself benign.

To assess bioactivity of the sCD14 being produced in the milk of the transgenic mice, a functional assay, based on stimulation of IL-8 secretion and cell detachment and lysis was conducted. Expression of IL-8 in test cultures wells in which no CD14 was added in the presence or absence of LPS was undetectable. The same was true for protein loss assay. Sample wells with no CD14 had no apparent loss protein; however, both IL-8 production and protein loss were apparent when milk from non-transgenic mice was added to LPS containing wells (Table 1) demonstrating the presence of endogenous sCD14 in milk of mice. Milk from transgenic animals approximately doubled the concentration of IL-8 measured; however, the IL-8 response was independent of the level of sCD14 in the transgenic milk, suggesting that the lowest expressing line produced sufficient amounts of sCD14 to reach a threshold that saturated the cellular response. A similar observation was made by monitoring protein loss, a measure of cell lysis, in the cultures. Cells incubated in PBS for the 24 hours of the test period averaged $162 \pm 77\,\mu g/mL$ of media. Addition of milk from nontransgenic mice reduced the protein content by approximately $47\,\mu g/mL$, while the milk from transgenic mice reduced the protein content of the wells by approximately $73\,\mu g/mL$. These observations clearly demonstrate there are endogenous constituents in mouse milk that have the ability to detach and or lyse cells in this assay. Presumably the IL-8 secretion and protein loss reflects endogenous sCD14. The endogenous activity was boosted by the presence of the transgene product, and, therefore, confirms the transgenic animals were producing biologically active sCD14.

Our working hypothesis predicted that increasing the immune surveillance of mammary glands by increasing the level of sCD14 in milk would reduce the severity of the infection by enhancing host detection and clearance of *E. coli* before the pathogen had a chance to multiply from a few bacteria to a population in the millions. To test our theory we purposely infused a very small number of *E. coli* into each challenged gland. Our target number was 5 CFU per gland. Culture of the solution infused revealed the actual bacterial concentration to be slightly higher than intended, 30 ± 1 CFU per gland. Remarkably, when infused glands were harvested 18 hrs postinfusion, glands averaged more than 100×10^6 CFU; thus, the strategy of predominant overexpression of sCD14 by mammary epithelial cells was inadequate to stem the logarithmic growth of *E. coli* in the infused glands. Mice from different lines responded similarly to bacterial growth. Mice from line 04, the highest expressing line, and 25, the lowest, had more bacteria in glands from transgenic animals then from nontransgenic littermates; whereas mice from the other two lines had approximately the same number of bacteria recovered from transgenic and nontransgenic glands. The lack of an association of response to different lines, differing in levels of expression, implies the additional sCD14 was inadequate, at least at the levels tested, to reduce bacterial growth. It is also possible that the hoped for response was masked by the wide variability in the parameter measured, recovered CFU. In another study investigating the effect of expression of an antimicrobial, lysostaphin, on the severity of mastitis, bacterial growth inhibition in transgenic mice expressing the gene was obvious and directly proportional to the level of expression of the transgene (9). The response in the current study might be expected to be somewhat different because the bacteria need to be first recognized by the innate immune system before host-derived antimicrobial responses can be activated. It would seem,

based on the bacterial recovery, that the triggering mechanism was not sufficiently sensitive or the response it elicited was inadequate to control bacterial growth.

The response of the three cytokines to *E. coli* challenge was similar and lends support to the lack of efficiency of exogenous sCD14. Levels of TNF-α, KC and MIP-2 were all elevated in response to *E. coli* infusion, ranging from 26 fold 480 increase (TNF-α) to a 230 fold increase (MIP-2); however, it was not possible to distinguish a difference in response due to genotype or line from which the mice were derived. The lack of a difference in responses by the different lines suggests that a 10-fold difference in levels of expression was not sufficient to diminish the innate immune response; however, the amount of gland edema clearly demonstrated a dose response relationship between sCD14 expression and an immunological response to *E. coli* infection. The weight of glands dissected 18 hours postinfusion was inversely correlated with the concentration of sCD14 produced by the mice of the four lines (Figure 2). The data closely fits an exponential decay function. The shape of the curve suggests that sCD14 expression levels above approximately 75 μg/mL should reduce susceptibility of edema in challenged animals. This interpretation of wet weight data is further supported by the difference between wet and dry weights. This measure of the fluid content of the glands confirmed infused glands were more edematous than noninfused glands and infused glands of transgenic animals were less edematous than glands from nontransgenic mice; thus, by this measure, it would appear that the presence of elevated sCD14 did reduce the severity of the immune response.

In summary, the current study investigated the effects of overexpression of sCD14 on the innate immune response to *E. coli* intramammary infection. The restricted overexpression of sCD14 by mammary epithelial cells did not enhance early mammary gland responses to *E. coli* as demonstrated by equivalent proinflammatory cytokine production by both the control and transgenic mice. Correspondingly, equivalent levels of bacteria were recovered from these mice; however, increased mammary production of sCD14 reduced infection-associated edema, presumably through the ability of sCD14 to neutralize LPS and/or restrict LPS access to membrane bound CD14. Thus, increased levels of sCD14 play a protective role during the innate immune response to intramammary infection by reducing inflammatory-mediated breakdown of the blood-milk barrier and corresponding edema formation.

REFERENCES

1. Schukken YH, van de GD, Grommers FJ, Smit JA, Brand A. Intramammary Infections and risk factors for clinical mastitis in herds with low somatic cell counts in bulk milk. Vet Rec 1989; 125:393–396.
2. Wurfel MM, Hailman E, Wright SD. Soluble CD14 acts as a shuttle in the neutralization of lipopolysaccharide (LPS) by LPS-binding protein and reconstituted high density lipoprotein. J Exp Med 1995; 181:1743–1754.
3. Lee JW, Paape MJ, Zhao X. Recombinant bovine soluble CD14 reduces severity of experimental Escherichia coli mastitis in mice. Vet Res 2003; 34:307–316.
4. Lee JW, Paape MJ, Elsasser TH, Zhao X. Recombinant soluble CD14 reduces severity of intramammary infection by Escherichia coli. Infect Immun 2003; 71:4034–4039.

5. Lee JW, Paape MJ, Elsasser TH, Zhao X. Elevated milk soluble CD14 in bovine mammary glands challenged with Escherichia coli 530 lipopolysaccharide. J Dairy Sci 2003; 86:2382–2389.
6. Paape M, Mehrzad J, Zhao X, Detilleux J, Burvenich C. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. J Mammary Gland Biol Neoplasia 2002; 7:109–121.
7. Ferrero E, Jiao D, Tsuberi BZ, Tesio L, Rong GW, Haziot A, Goyert SM. Transgenic mice expressing human CD14 are hypersensitive to lipopolysaccharide. Proc Natl Acad Sci USA 1993; 90:2380–2384.
8. Tamura Y, Higuchi Y, Kataoka M, Akizuki S, Matsuura K, Yamamoto S. CD14 transgenic mice expressing membrane and soluble forms: comparisons of levels of cytokines and lethalities in response tolipopolysaccharidebetween transgenic and non-transgenic mice. Int Immunol 1999; 11:333–339.
9. Kerr DE, Plaut K, Bramley AJ, Williamson CM, Lax AJ, Moore K, Wells KD, Wall RJ. Lysostaphin expression in mammary glands confers protection against staphylococcal infection in transgenic mice. Nat Biotechnol 2001; 19:66–70.
10. Wall RJ, Powell A, Paape MJ, Kerr DE, Bannerman DD, Pursel VG, Wells KD, Talbot N, Hawk HW. Genetically enhanced cows resist intramammary Staphylococcus aureus infection. Nat Biotechnol 2005; 23:445–451.
11. Simons JP, McClenaghan M, Clark AJ. Alteration of the quality of milk by expression of sheep beta- lactoglobulin in transgenic mice. Nature 1987; 328:530–532.
12. Simons JP, Wilmut I, Clark AJ, Archibald AL, Bishop JO. Gene transfer into sheep. Bio/Technology 1988; 6:179–183.
13. Wang Y, Zarlenga DS, Paape MJ, Dahl GE. Recombinant bovine Soluble CD14 sensitizes the mammary gland to lipopolysaccharide. Vet Immunol Immunopathol 2002; 86:115–124.
14. Maga EA, Anderson GB, Murray JD. The effect of mammary gland expression of human lysozyme on the properties of milk from transgenic mice. J Dairy Sci 1995; 78:2645–2652.
15. Nemchinov LG, Paape MJ, Sohn EJ, Bannerman DD, Zarlenga DS, Hammond RW. Bovine CD14 receptor produced in plants reduces severity of intramammary bacterial infection. FASEB J 2006; 20:1345–1351.
16. Jara-Almonte M, White JM. Milk production in laboratory mice. J Dairy Sci 1972; 55:1502–1505.
17. Mitra A, Hruska KS, Wellnitz O, Kerr DE, Capuco AV, Wall RJ. Expression of lysostaphin in milk of transgenic mice affects the growth of neonates. Transgenic Res 2003; 12:597–605.
18. Filipp D, izadeh-Khiavi K, Richardson C, Palma A, Paredes N, Takeuchi O, Akira S, Julius M. Soluble CD14 enriched in colostrum and milk induces B cell growth and differentiation. Proc Natl Acad Sci USA 2001; 98:603–608.
19. Snijders BEP, Damoiseaux JGMC, Penders J, Kummeling I, Stelmaf F, Van Ree R, et al. Cytokines andsoluble CD14 in breast milk in relation with atopic manifestations in mother and infant (KOALA study). Clinical and Experimental Allergy 2001; 36:1609–1615.
20. Stein T, Morris JS, Davies CR, Weber-Hall SJ, Duffy MA, Heath VJ, Bell AK, Ferrier RK, Sandilands GP, Gusterson BA. Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14, and STAT3. Breast Cancer Res 2004; 6:R75–R91.
21. Zheng J, Watson AD, Kerr DE. Genome-wide expression analysis of lipopolysaccharide-induced mastitis in a mouse model. Infect Immun 2006; 74:1907–1915.

22. Archibald AL, McClenaghan M, Hornsey V, Simons JP, Clark AJ. High-level expression of biologically active human alpha 1-antitrypsin in the milk of transgenic mice. Proc Natl Acad Sci USA 1990; 87:5178–5182.
23. Yull F, Harold G, Wallace R, Cowper A, Percy J, Cottingham I, Clark AJ . Fixing human factor IX (fIX): Correction of a cryptic RNA splice enables the production of biologically active fIX in the mammary gland of transgenic mice. Proc Natl Acad Sci USA 1995; 92:10899–10903.
24. Burdon T, Wall RJ, Shamay A, Smith GH, Hennighausen L. Over-expression of an endogenous milk protein gene in transgenic mice is associated with impaired mammary alveolar development and a milchlos phenotype. Mech Dev 1991; 36:67–74.